



Changes in expression of polyamines and ethylene biosynthesis genes in groundnut (*Arachis hypogaea* L.) genotypes during *Sclerotium rolfsii* infection

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Stem rot disease caused by fungal pathogen, *Sclerotium rolfsii* Sacc., is potential threat to groundnut production in warm and humid condition. After host-pathogen interaction, a multitude of plant resistance associated reactions are initiated. In the present investigation we studied the role of polyamines and ethylene during host-pathogen interaction in stem rot tolerant (CS319, GG17 and GG31) and susceptible (TG37A) groundnut genotypes at 24, 48 and 72 h after infection. Stem rot tolerant genotypes showed higher expression of polyamine biosynthesis genes ornithine decarboxylase (*Ordec*), spermine synthase (*Sms*) and lipoxygenase1 (*LOX1*) gene at 72 h after infection than that of susceptible genotype TG37A. The expression analysis of ethylene biosynthesis genes (1-aminocyclopropane-1-carboxylate oxidase: *ACC1* and (*ACC2*) showed up regulation in stem rot susceptible genotype TG37A than that of tolerant genotypes after infection at all stages (24, 48 and 72 h after infection). The expression of amine oxidase (*AMO*) gene was observed highest in stem rot susceptible genotype TG37A while minimum in GG31. Expression of this gene was remarkably induced in TG37A which may leads to higher accumulation of H₂O₂. Higher content of a polyamine, putrescine was found in the leaves of stem rot tolerant genotypes at 48 and 72 h after infection. These results implied that tolerant genotypes induced higher polyamine biosynthesis which may involve in plant defense and impart tolerance/ resistance. While, susceptible genotype (TG37A), utilized higher flux of S-Adenosyl methionine (SAM) for ethylene biosynthesis which may leads to necrosis of plants. Thus, stem rot resistant genotypes may be developed through genetic manipulation of polyamine biosynthesis pathway.

Keywords: Fungal, host-pathogen interaction, Stem rot disease

Groundnut (*Arachis hypogaea* L.) is an important oilseed legume, mostly grown in arid and semi-arid regions of the world. India is one among the leading producers of groundnut in the world. However, productivity of the crop is very low (1422 kg/ha) as compared to the leading producer China (3893 kg /ha) and USA (4426 kg/ha)¹ mainly because of various biotic and abiotic stresses. Among the biotic stress, stem rot diseases, caused by facultative parasitic fungi, *Sclerotium rolfsii* Sacc., is potential threat to groundnut production in warm and humid condition. The disease is prevailed at 30 to 45 days after germination and at the time of harvest under rain-fed situations due to low and erratic distribution of rainfall. Generally, fungus attacks stem of groundnut plants near the soil surface however, in the light soil, it also damage pegs and pods². Due to wide host range, soil borne nature, late diagnosis of disease³,

and rapid multiplication of the pathogen development, use of the resistant variety is only feasible solution of the problem⁴.

Diverse regulatory processes mediate host resistance response to pathogen infection, of which, plant hormone functions have been studied extensively⁵. Upon the perception of the *S. rolfsii* in groundnut, the jasmonic acid-mediated defense pathway gets activated, thereby inducing systemic resistance in groundnut⁶. In tomato, ethylene was imparting resistance against necrotrophic fungi *Botrytis cinerea*⁷. An early and active response of plants to perception of pathogen attack is production of ethylene which is associated with the induction of defense reactions. However, contradictory results have been obtained in different conditions and the plant-pathogen combination⁸. In response to many virulent pathogen infections in plants, ethylene evolution occurs concomitantly with the progression of disease symptoms⁹. Putrescine, spermidine, and spermine are the three most important polyamines in

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plants. In both compatible and incompatible plant-microbial interactions, appreciable changes in polyamines metabolism have been observed¹⁰. Putrescine and spermidine content increased in maize (*Zea mays*) plants after infection with the fungal biotroph *Ustilago maydis*¹¹. Similarly, six-fold increase in spermidine content observed in barley (*Hordeum vulgare*) leaves infected with the brown rust fungal biotroph *Puccinia hordei* compared to control healthy leaves and resulted in the formation of green islands surrounding the site of infection due to chlorophyll retention¹². In contrary, a decrease in content of polyamine has been observed in tobacco leaves inoculated with fungal, bacterial and viral pathogens¹³. Biosynthesis of putrescine leads by decarboxylation of ornithine by ornithine decarboxylase or arginine by arginine decarboxylase through agmatine, subsequently putrescine is converted to spermidine, and spermine by spermidine synthase and spermine synthase enzyme respectively¹⁴. S-adenosyl-methionine (SAM) is a common precursor for both polyamine and ethylene¹⁵. In tomato fruits, ethylene induced by *Rhizopus stolonifer* infection was reported to be responsible for the decreased content of polyamine¹⁶. Thus, the rate of biosynthesis of both molecules regulates expression of each other, which is a decisive factor in determining the predominance of either of these two pathways during different conditions¹⁷.

Polyamine oxidase and lipoxygenase enzymes are key players for hypersensitive reaction (HR) during host-pathogen interaction¹⁸. As a signal molecule, H₂O₂ derived from polyamine oxidation mediates cell death, the hypersensitive response and the expression of defense genes¹⁹. Lipoxygenase is able to generate different functional molecules that include (a) hydroperoxides and free radicals that might be involved in the localized cell death observed during the hypersensitive response; (b) jasmonic acid and its methyl ester that can trigger defense gene expression; and (c) 2-trans-hexenal acts as antimicrobial compounds for direct defense against pathogen attack²⁰.

In this context, we studied the expression of five different genes *viz.*, ornithine decarboxylase, spermine synthase, ethylene biosynthesis, lipoxygenase1, amine oxidase along with polyamine profiling to elucidate the role of polyamines and ethylene in the reaction of different groundnut genotypes to the infection of facultative parasitic fungi, *Sclerotium rolfsii*.

Materials and Methods

Plant material and growth conditions

Seeds of one stem rot susceptible groundnut genotype TG37A and three Stem rot tolerant genotypes CS319, GJG17 and GJG31 obtained from ICAR- Directorate of Groundnut, Junagadh, India. These genotypes were selected based on previous reports on stem rot incidence^{4,21}. Seeds were sown in earthen pots (20 kg soil capacity) in PII glass house under controlled atmospheric conditions. A potting mixture consisting Vertisol, sand and farm yard manure in a 2:1:1 ratio with diammonium phosphate at 1.0 g kg⁻¹ of soil was used. Each genotype was grown in six pots, three pots were kept for control and three for inoculation of stem rot pathogen.

Inoculation of *S. rolfsii* in groundnut and sample collection for analysis

S. rolfsii was isolated from TG-37A genotype and cultured in 90 mm Petri dishes containing standard Potato Dextrose Agar (PDA) medium. The fungal pathogen *S. rolfsii* was further mass multiplied on sorghum grains. The fungus multiplied in sorghum grain was inoculated to about 45 days old groundnut plant by placing infested sorghum grain (about 2 g) on soil surface near the main stem. For ensuring the developing of mycelia, the experiment was carried out in controlled condition with natural photoperiod (temperature 30±2°C relative humidity above 70% and soil moisture content near field capacity) in glass house²³. The pathogen was re-isolated to prove Koch's postulates immediately after the expression of symptoms. Disease symptoms were visually appeared after 48 h (Fig. 1). Leaf samples were collected from infected and non-infected genotypes at 24, 48 and 72 h after infection because hyphae from germinating sclerotia of *S. rolfsii* ramified over host tissue within 24-48 h following inoculation²². Second upper leaves were collected from 3 replications of plants at each time interval. Leaf samples used for polyamines analyses were stored at -20°C.

RNA extraction, quantification quality check and cDNA Synthesis

Fresh leaves (0.1 g) were collected at 24, 48 and 72 h after infection from infected and non-infected plants of each genotype. Total RNA was extracted from the leaves by a TRIZOL (Invitrogen) reagent as per standard protocol. The RNA samples with the ratio of 1.7-2.0 at OD 260/280 were used for cDNA synthesis. Agarose gel (0.8% w/v) was prepared in 1.0 X formaldehyde agarose gel buffer and used to check

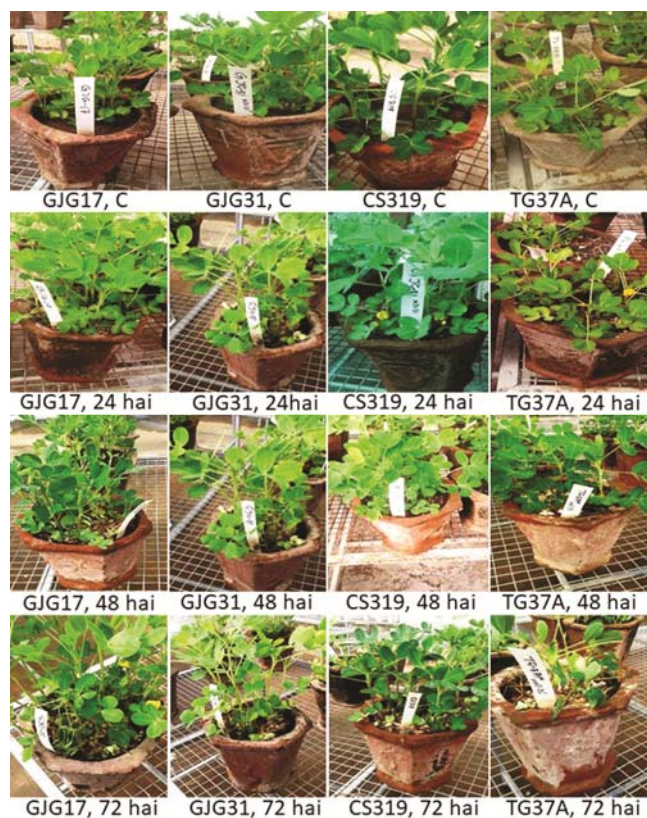


Fig. 1 — Stem rot tolerant (GJG17, GJG31 and CS319) and susceptible (TG37A) groundnut genotypes at 0 (Control), 24, 48 and 72 h after infection. Visual symptoms of disease appeared after 48 h of infection

RNA quality. The cDNA was synthesized using RevertAid™ First Strand cDNA Synthesis Kit (The Thermo Scientific™) following manufacturer's instructions.

Primers for Real Time PCR

Gene sequences for polyamine and ethylene biosynthesis genes along with amine oxidase and lipoxygenase gene of groundnut were searched on NCBI. The primers were designed by using NCBI-primer 3 software (Table 1), while for endogenous control actin primer sequences reported by Morgante *et al.*²³ were used.

Quantitative RT-PCR analysis

PCR was performed using 1.0 µL (≤100 ng/reaction) aliquot of the first strand cDNA in a final volume of 10 µL containing 10 pM of specific primers (forward and reverse). As an endogenous control, the groundnut actin primer²³ was used to normalize each sample for variations in the amounts of RNA used. PCR was carried out using 5 µL 2x QuantiFast SYBR Green PCR Master Mix (Genetix,

Table 1 — List of genes and their primer sequence used for expression analysis

Name of gene	Accession no.	Primer (R- Reverse, F-Forward)
Ornithine decarboxylase (<i>OrDec</i>)	GT 735346.1	CATGGAGAATAATCAAAGGATCTTC R AAGGCATGTACTGTCAAGCCAC F
ACC oxidase 2 (<i>ACC2</i>)	EE 125763.1	CCTCAAATGTCCAAACCCAAAG R GCCTTCCAACCTTTGTCATCTTG F
ACC synthase (<i>ACCS</i>)	EE 125903.1	TGAATTCAGTACCCATCAGG R TCCTCCCTATGTTCTTCTAGGT F
Spermine synthase (<i>Sms</i>)	GT 735283.1	GTCTAACTTCGGGCTTGCT R CCCTCACACCCTTCTCTAA F
Amine oxidase (<i>Amo</i>)	EE 123718.1	GTTTCAGCCATTTCCGATCTTG R AACAATGCCAGGAGTTCTAC F
Lipoxygenase 1 (<i>LOXI</i>)	AF 231454.1	CAGTATCCTTATGGAGGGCTATC R GGTGACTCTTACCATCTCTTC F
Actin	Morgante <i>et al.</i> ²⁴	GAGCTGAAAGATTCCGATGC R GCAATGCCTGGGAACATAGT F

USA), both reverse and forward primer were 0.5 µL, Template cDNA 1.0 µL and RNase free water 3 µL to final volume 10 µL in a thermal cycler (ABI-7300) programmed as follows. An initial denaturation for 5 min at 95°C, 35 amplification cycles [5 min 95°C (initial denaturation), 10 s at 95°C (denaturation), 30 s at 58°C/60°C (annealing)] for primers. Each sample was analysed in triplicate for all primers. To ensure amplification of a single product with the expected melting temperature and the absence of primer-dimers a melting curve analysis was performed on all samples. The products of each primer set were observed using agarose gel (0.7%) electrophoresis. To estimate relative gene expression, Ct value of both reference and target genes were calculated based on the mean value of three replications. Relative RNA quantities were determined with delta-delta ($\Delta\Delta$) Ct, using the formula given by Livak and Schmittgen²⁴.

HPLC analysis for polyamines

Polyamines were analyzed through HPLC as described by Flores and Galston²⁶ with minor modifications. Polyamines were extracted from leaves in 5 % cold perchloric acid (HClO₄) at a ratio of about 200 mg/2mL HClO₄. Extracted samples were incubated for 1.0 h in an ice bath and centrifuged at 15000 rpm for 20 min. Supernatant containing the free polyamine fraction was collected in Eppendorf tube (1.5 mL) and stored at -20°C till further analysis.

Sample preparation (Benzoylation)

Plant extracts and standards (500 µL) were benzoylated using 1.0 mL of 2 N NaOH and 10 µL benzoyl chloride as per method described by Redmond and Tseng²⁶. Benzoylated polyamines were extracted in 2.0 mL diethyl ether by centrifugation at

3000 rpm for 5 min. Ether phase containing polyamines was collected in 1.5 mL eppendorf tube and dried in a vacuum concentrator at 40°C. These dried polyamines were redissolved in 100 μ L methanol (HPLC grade). Standards were treated in a similar way, with up to 100 ppm of each polyamine in the reaction mixture. The benzoylated samples were stored at -20°C till further analysis. The isocratic solvent system consisted methanol: water run at 60% at the flow rate of 1.0 mL/min. The benzoylated extracts were eluted at room temperature (25 \pm 1°C) using a C18 reverse- phase column (4.6 \times 250 mm, 5 μ m particle size) and detected at 254 nm. The quantity of individual polyamines was calculated based on area and concentration of standards.

Statistical analysis

Quantitative qPCR data were statistically compared between non-infected and infected samples at each time using two factor (genotype \times time) factorial analysis and values were compared $P < 0.05$ for significance. Data processing and time series analysis of polyamines was carried out using MetaboAnalyst 5.0, an online statistical package developed by Pang *et al.*²⁸.

Results and Discussion

Expression of ornithine decarboxylase and spermine synthase gene

Significant differences in the expression of polyamine and ethylene biosynthesis genes were

observed at 72 h after infection. While at 24 and 48 h after infection no distinguish expression pattern of polyamine and ethylene biosynthesis genes were observed in susceptible and tolerant genotype. The expression of ornithine decarboxylase (*Ordec*) gene was higher in stem rot tolerant groundnut genotypes (GJG17, GJG31 and CS319) than that of susceptible genotype TG37A at 72 h after infection (Fig. 2A). Among the different tolerant genotypes, highest expression of *Ordec* gene was observed in GJG31 followed by CS319 and GJG17 at 72 h after infection. Expression of spermine synthase (*Sms*) gene was increased with progression of disease in all genotypes but CS319 had appreciably higher expression than other genotypes at 72 h after infection (Fig. 2B). The expression of *Sms* gene in stem rot tolerant genotype CS319 (18.79 fold) was more than 2 fold as compared to susceptible genotype TG37A (8.10 fold) at 72 h after infection. Higher expression of *Ordec* was further supported by higher putrescine content as shown in polyamine profiling data. Ornithine decarboxylase synthesizes diamine putrescine that is the precursor for the more complex triamine spermidine and tetraamine spermine. The enzymes spermidine synthase and spermine synthase have a key role in polyamine biosynthesis²⁸. In tolerant genotype *Ordec* is activated and synthesized putrescine. The results indicate that the activation of *Ordec* is critical while activation of *Sms* is situational and time dependent.

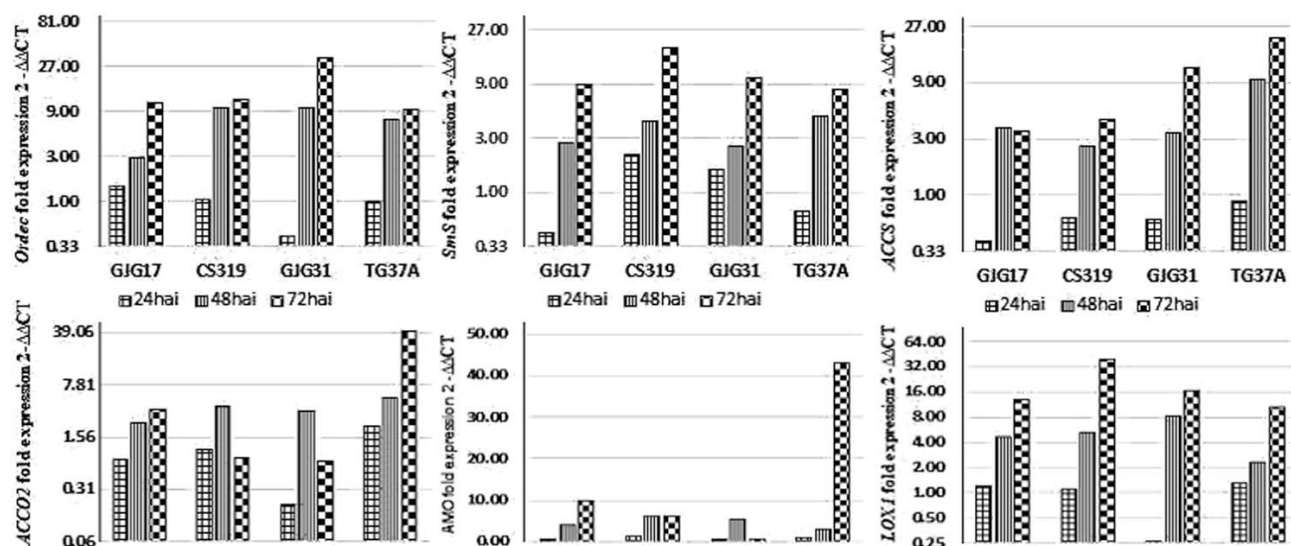


Fig. 2 — Comparative fold change expression of genes in groundnut genotypes in response to *Sclerotium rolfii* infection at 24, 48 and 72 h time interval. Data were analyzed by two factor (genotype \times time) factorial experiment and values were compared $P < 0.05$ for significance (A) ornithine decarboxylase (*Ordec*), $P < 0.05$: 0.335; (B) spermine synthase (*Sms*) genes, $P < 0.05$: 0.49; (C) 1-aminocyclopropane-1-carboxylate synthase (*ACCS*), $P < 0.05$: 0.425; (D) 1-aminocyclopropane-1-carboxylate oxidase, $P < 0.05$: 662; (E) lipoxigenase 1 (*LOX1*), $P < 0.05$: 0.629; and (F) amine oxidase (*AMO*) gene, $P < 0.05$: 0.859.

Thus higher expression of polyamine biosynthesis genes in stem rot tolerant genotypes resulted in higher polyamine accumulation particularly putrescine which exhibited enhanced tolerance to stem rot pathogen. Similarly, reductions in the activities of ornithine decarboxylase and arginine decarboxylase were also observed in tomato infected with the fungus *Rhizopus stolonifer*, and these changes were accompanied by reduced polyamine concentrations¹⁶. Overexpression of heterologous *adc*²⁹ or *ordc* cDNAs leads to elevated putrescine levels in plant³⁰.

Expression analysis of ethylene biosynthesis genes

The expression of 1-aminocyclopropane-1-carboxylate synthase (ACCS) gene was observed higher in stem rot susceptible genotype TG37A at all time intervals (Fig. 2C). Tolerant genotype (GJG 17 and CS319) showed lower expression compared to TG37A. The expression of 1-aminocyclopropane-1-carboxylate oxidase 2 (ACCO2) gene was lower at early stage, but stem rot susceptible genotype (TG37A) exhibited highest expression at later stages of pathogen attack. On the other hand, tolerant genotype (CS319 and GJG31) showed significantly less expression than that of TG37A and GJG17 (Fig. 2D). These results clearly differentiate tolerant and susceptible genotypes based on ACCO expression pattern. At 48 h after infection, all genotypes showed little expression of ACCO2 gene which might start signals for defense reaction but higher expression of ACCS and ACCO2 genes may leads to sudden burst of ethylene in TG37A at 72 h after infection and subsequent necrosis, senescence and death of plants. Our results suggested that a sub-optimal level of ethylene may require initiating ethylene mediated defense reactions but higher levels may cause plant death.

Increased ethylene evolution was also observed in tomato plant infected with *Rhizopus stolonifer*³¹. These results are further supported by observation of Brown and Lee³²; they observed that ethylene production induces susceptibility to stem-end rot of citrus. A significant increase in ACCO gene expression was also observed in the *Xanthomonas campestris* pv. *vesicatoria* challenged tomato plants³⁴. Ethylene mediated disease reaction was further confirmed by incorporation of ACC deaminase producing bacteria, *Paenibacillus lentimorbus* B-30488 in soil. These bacteria ameliorated deleterious enhanced ethylene level in *S. rolfii* infected tomato plants. Modulated activities of ACCO and ACCS were

also observed in *S. rolfii* infected plants. These results concluded that ACC deaminase producing bacteria (B-30488) diminishing the ethylene production thereby control the southern blight disease caused by *S. rolfii*³⁴. Since the pathways for the biosynthesis of polyamines and ethylene share a common precursor S-adenosyl-methionine (SAM), an increase in ethylene evolution should, theoretically, lead to a reduction in polyamine biosynthesis. These results suggest complex regulation of SAM homeostasis³⁵. New insights also placed SAM homeostasis and transmethylation in relation to promote plant virus infections, during which biosynthesis of ethylene is also important³⁶. Moreover, overexpression of ethylene may inhibit jasmonic acid mediated defense response³⁷ which may lead to stem rot disease induction in susceptible genotypes.

Expression analysis of lipoxygenase1 (LOX1) and amine oxidase (AMO) genes

LOX1 expression induced with time dependent manner in all genotypes. However, highest expression of LOX1 gene observed in stem rot tolerant genotype CS319 at 48 and 72 h after infection (Fig. 2E). While, in susceptible genotype (TG37A) LOX1 expressed 4 times lesser than that of CS319. Similarly, in groundnut, the gene coding for PnLOX1 is induced in mature seeds infected with *Aspergillus* spp. The products of reactions catalyzed by PnLOX1 conferred a role in plant-fungus interaction to this particular lipoxygenase. The PnLOX1 product namely (13S)-hydro-peroxy-(9Z,11E)-octadecadienoic (13-HPOD) is an inhibitor and (9S)-hydroperoxy-(10E, 12Z)-octadecadienoic acid (9-HPOD) is an inducer of mycotoxin synthesis³⁸.

Moreover, overexpression of pepper *CaLOX1* gene in *Arabidopsis* (*Arabidopsis thaliana*) conferred enhanced resistance to *Alternaria brassicicola*, *Pseudomonas syringae* pv *tomato* and *Hyaloperonospora arabidopsidis*. In contrast, mutation of the *Arabidopsis CaLOX1* ortholog *AtLOX1* significantly increased susceptibility to these three pathogens. Together, these results suggest that *CaLOX1* and *AtLOX1* positively regulate defense and cell death responses to microbial pathogens³⁹. The remarkable involvement of *CaLOX1*, *AtLOX1* and *RcLOX5*⁴⁰ in mediating resistance to pathogen attack suggests that these LOX genes are highly conserved for disease resistance in plants. Plant LOXs have been proposed to play a role in gene activation during wound response and necrotrophic fungal pathogen infection⁴⁰. Higher expression of LOX1 gene in stem rot tolerant genotypes further confirmed the role of

lipoxygenase in disease resistance. Higher expression of LOX may contribute for jasmonic acid signaling pathway⁴¹ which was identified as a possible defense mechanism in groundnut against stem rot disease⁶.

Highest upregulation of amine oxidase (*AMO*) gene was observed in stem rot susceptible genotype TG37A while minimum in GJG31 at 72 h after infection. *AMO* gene expression was observed higher in tolerant genotypes at 48 h after infection, which further increased in all genotypes at 72 h after infection. However, expression of this gene was remarkably induced (about 4.5 fold) in TG37A at 72 h after infection (Fig. 1F). Polyamine oxidase is important in producing H_2O_2 *in-vivo* during host-pathogen interaction, cell growth and differentiation⁴²⁰. H_2O_2 is a mediator of several physiological events such as

lignification and wall stiffening and programmed cell death. In present investigation, higher expression of PAO gene in stem rot susceptible genotype (TG37A) may cause more accumulation of H_2O_2 which leads membrane leakage and ultimately plant death. These results suggest that excess expression (beyond a threshold limit) of *AMO* and ethylene biosynthesis genes may harmful during *S. rolfisii*- groundnut interaction, however optimal level of *AMO* and *ACCS* and *ACCO* expression is essential for H_2O_2 and ethylene mediated defense reactions.

Polyamine profiling

Agmatine and putrescine content was increased in the leaves of all groundnut genotypes at 24 h after infection but concentration of agmatine was higher in stem rot susceptible genotype TG37A (Fig 3).

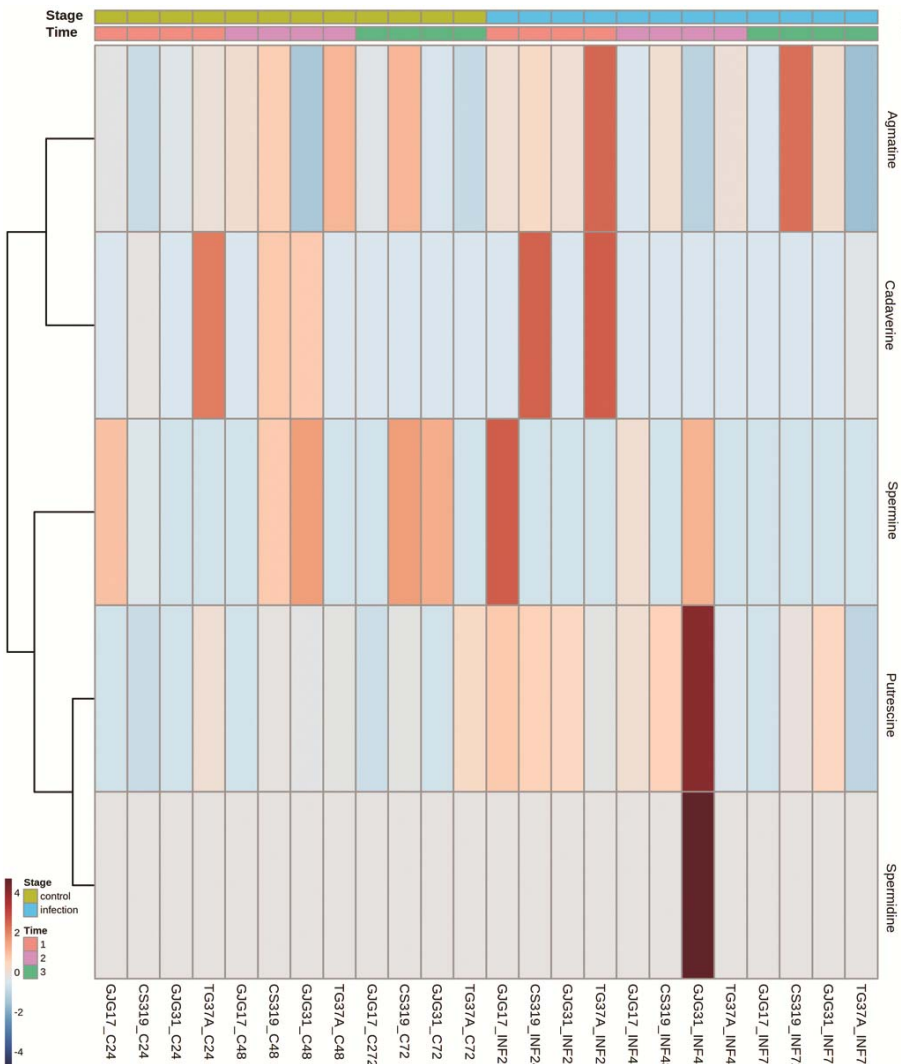


Fig. 3 — Heat map of polyamine in groundnut genotypes during *Sclerotium rolfisii* infection at 24, 48 and 72 h time interval. [Time series analysis was done using MetaboAnalyst 5.0 online software where C= control, INF= infected]

Constitutive level of putrescine and cadaverine also observed higher in TG37A. Cadaverine was increased at 24 h after infection in TG37A. However, TG37A could not maintain the level of any polyamines except agmatine at later stages of infection (48 and 72 h after infection). On the other hand, concentration of agmatine and putrescine increased in the stem rot tolerant genotypes (GJG17, GJG 31 and CS 319) at 48 and 72 h after infection. Though, the levels of polyamines were varied in genotype to genotype. Interestingly, spermine was not observed in TG37A at any stage of analysis. However, spermidine was detected only in GJG31 at 48 h after infection.

These results of polyamines profiling are supported by expression analysis of arginine decarboxylase and spermine synthase gene. Furthermore, our results are corroborated with a previous report where increased resistance against *Fusarium* wilt was observed in over-expressing arginine decarboxylase gene in transgenic eggplant⁴³. Higher spermine content was also reported after *Fusarium* infection in wilt resistant genotypes of castor¹⁸. Higher content of spermine plays a role as a mediator in defense signaling against wilt disease caused by *Fusarium oxysporium*⁴³ and tobacco mosaic virus⁴⁴. Therefore, higher levels of polyamines in resistant genotypes after infection (48 h) suggest their involvement in fungal disease resistance.

Conclusion

This study generated some basic understanding of the signals mediated defense mechanism of groundnut against *Sclerotium rolfsii* infection. These results implied that tolerant genotypes induced higher polyamine biosynthesis which may involve in plant defense and impart resistance/tolerance. While in susceptible genotype (TG37A), excess expression of ethylene biosynthesis genes at 72 h after infection may utilize higher flux of S-adenosyl-methionine for ethylene biosynthesis instead of polyamine biosynthesis. Expression of hydroperoxides producing enzymes *LOX1* was higher in tolerant genotype leads to induce hypersensitive reaction. Higher expression of LOX may contribute in JA signaling pathway which has been recently identified as a possible defense mechanism in peanut against stem rot disease. However, higher expression of amine oxidase in susceptible genotype may exert oxidative stress and higher membrane-lipid peroxidation due to pathogen infection which ultimately cause plant death. Thus,

stem rot resistant genotypes may be developed through genetic manipulation of polyamine biosynthesis pathway.

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Conflict of interest

Authors declare no competing interests.

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